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Research Article

DNA metabarcoding reveals the influence of land cover and farming on the dietary composition of a spider-specialist bat

Nerea Vallejo[1], Miren Aldasoro[1], Lander Olasagasti[1], Joxerra Aihartza[1], Inazio Garin[1]

1 *Department of Zoology and Animal Cell Biology, University of the Basque Country UPV/EHU, Sarriena z.g., 48940, Leioa, The Basque Country, Spain*
Corresponding author: Nerea Vallejo (nerea.vallejo@ehu.eus)

Abstract

Human-driven landscape transformations are the primary factors driving biodiversity changes in the present century. For insectivorous bats, land-use modifications and intensification of agriculture negatively impact bat foraging activity and, consequently, the potential ecosystem services they can provide. However, little is known about specific dietary niche adaptations to anthropogenic land changes. Here, we analysed the influence of land cover and agricultural practices, especially livestock, on the diet of the notch-eared bat. We collected faecal samples from sixteen maternity colonies in the Iberian Peninsula and southern France during two different sampling periods, May and July of 2020. We analysed 318 faecal samples using DNA metabarcoding, resulting in 241 identified prey species. Spiders were the most consumed prey item, followed by livestock pest flies (Muscidae). Dietary diversity was higher in May, while the weighted percentage of occurrence (wPOO) of spiders was higher in July. Consumption of harmful livestock pests was more likely in areas where livestock were prevalent near the colony; however, other landscape characteristics such as forest cover and urban areas had a greater influence on the wPOO values of pests. Our results highlight the consistency of the dietary composition of the notch-eared bat across large geographical areas, and its reliance on gleaning for hunting motionless prey. The interaction between livestock farming densities, livestock management types and land use around the colony will influence the densities of pest flies and other arthropods, ultimately shaping the trophic niche of *M. emarginatus*.

Key words: Adaptations, Araneidae, filth flies, high throughput sequencing, human altered environment, livestock pest, *Myotis emarginatus*

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Introduction

Anthropogenic transformations of landscapes and land use are the primary factors driving changes in biodiversity for the present century (Sala et al. 2000). The replacement of natural areas with agricultural fields, intensive forestry plantations, pastures, and urban areas has been the major exponent of land use changes in the past centuries (Foley et al. 2005). In fact, heavily modified and ecologically degraded land is causing population declines in almost half the species worldwide (Finn et al. 2023), including insects, which has led to a cascade effect on their predators, for example birds or bats (Park 2015; Forister et al. 2019; Sánchez-Bayo and Wyckhuys 2019; Puig-Montserrat et al. 2021; Rigal et al. 2023).

Diet variability in bats is common and largely results from intrinsic differences in arthropod communities across different locations. This variability is supported by the bats' ability to consume a wide range of arthropod prey species (Clare et al. 2011; Tiede et al. 2020; Tournayre et al. 2020; Wray et al. 2020). Consequently, bats can adapt their dietary niche to changes in availability due to environmental stressors or human-altered environments (Jung and Kalko 2010; Maine and Boyles 2015).

However, the success of the dietary response will depend on the degree of specialization (Maine and Boyles 2015). Some bat species are regarded as generalist and opportunistic foragers, eating whatever prey they encounter within their morphoecological limitations (Divoll et al. 2022); while others actively select the most profitable prey items and use adaptive foraging strategies (Jones 1990; Arrizabalaga-Escudero et al. 2019). Species that have a broader trophic niche are more likely to persist in heavily modified landscapes (Maine and Boyles 2015) and face a lower risk of extinction (Boyles and Storm 2007).

Landscape modifications not only limit potential predator-prey interactions but may also enable new ones as species adapt to exploit new resources and foraging areas (Wong and Candolin 2015). For example, foraging around artificial lighting is well known among bats foraging in urban areas (Rydell and Racey 1995; Lewanzik and Voigt 2016; Stidsholt et al. 2023), but they are less successful compared to bats in rural areas as they rely less on eavesdropping conspecifics (Stidsholt et al. 2023). Many studies suggest that specialist insectivorous bats forage successfully in intensive culture areas (Baroja et al. 2021), where they forage on pests that are functionally similar to typical prey (e.g., Aizpurua et al. 2018; Mata et al. 2021). In such areas, the decline of prey diversity linked to agricultural intensification (Wickramashinghe et al. 2004) will likely affect bats' dietary diversity as well (Aizpurua et al. 2018).

Active feeding around cattle and cattle dung in open fields, or inside cattle sheds, is also well-known (Krull et al 1991; Downs and Sanderson 2010; Siemers et al. 2012; Ancillotto et al. 2017). The notch-eared bat (*Myotis emarginatus*) is an example of a species that often hunts in cattle sheds (Dietz and Pir 2021). This species is a medium-sized vesper bat that is distributed around Mediterranean Europe but occurs as far north as the Netherlands and Germany, eastwards to Tajikistan, and in some areas in the north of Africa (Piraccini 2016). Several radiotracking studies in Germany (Krull et al. 1991; Zahn et al. 2009), Luxembourg (Dietz et al. 2013; Pir and Dietz 2018) and the Netherlands (Dekker et al. 2013) have shown a close association between *M. emarginatus* and agricultural practices related to traditional cattle farming. They hunt inside cattle sheds, where they consume immobile diurnal flies, specifically stable flies (*Stomoxys calcitrans*) and house flies (*Musca domestica*) (Krull et al. 1991; Steck and Brinkmann 2006; Kervyn et al. 2012; Vallejo et al. 2023; Vescera et al. 2024). A recent study reported that the pest fly consumption does not correlate with livestock numbers around the colony, although regretfully, few colonies were surveyed and the range of cattle heads around them was limited (Vescera et al. 2024). Other studies, however, show that it forages mostly in native deciduous and riparian forests, where it preferentially consumes orb-web building spiders (Krull et al. 1991; Fonderflick et al. 2015), especially in Mediterranean locations (Goiti et al. 2011; Vallejo et al. 2019).

While it is generally regarded as an adaptable and synanthropic species (Dietz et al. 2013; Vescera et al. 2024), this capability is not explained by its dietary

breadth, which can be considered narrow compared to other European bat species (Alberdi et al. 2020; Mata et al. 2021). In fact, five nursing colonies in the Basque Country focused the bulk of their diet on no more than a dozen prey species throughout their short active season (Vallejo et al. 2023) and spiders and flies accounted for around 80% of the total prey volume. Occasionally, it has been reported to feed in abundance on other types of prey such as coleopterans (Benda et al. 2010; Vallejo et al. 2019), highlighting the species' potential for adaptability.

In this study, we aim to analyse how landscape and anthropogenic land use changes, especially livestock farming, impact the diet of the notch-eared bat at two different periods of the maternity season. We focus our study on the northern Iberian Peninsula and southern France, in the core distributional area of the western population of *M. emarginatus* (Frantz et al. 2022). We chose 16 colonies in areas with varied climatic conditions, from temperate oceanic to Mediterranean; and various associated types of anthropogenic landscape modifications. Based on previous studies, we expected to find variability in the diet composition of *M. emarginatus* between the colonies. While we expected diurnal pest flies to be a relevant part of the diet, we predicted that spiders would be the most abundant prey. Additionally, we chose colonies with varying degrees of livestock farming, ranging from none to widespread, and with different management practices, to explore the shift from a spider-based to a pest-fly-based diet. We expected the consumption of diurnal flies to be restricted exclusively to those colonies located in areas with the highest density of livestock.

Methods

Description of the study area

Sampling was carried out in 16 maternity colonies of *Myotis emarginatus*, encompassing various climatic profiles (Fig. 1), from temperate oceanic, characterized by moderate temperatures and wet summers, to Mediterranean, characterized by dry summers (Peel et al. 2007). We obtained precipitation data for the study area using WorldClim 2.1 (Fick and Hijmans 2017). The size of the colonies ranged from 30 to 800 individuals. All colonies were found in buildings, and in some of the colonies other bat species were present, such as *Rhinolophus ferrumequinum*, *Miniopterus schreibersii* or *Myotis capaccini* (Suppl. material 1).

Landscape characterization

To include the maximum potential foraging area of *M. emarginatus* (Flaquer et al. 2008; Goiti et al. 2011; Dietz et al. 2013), the landscape around the colonies was characterized in a 10 km radius around the colonies. We used 10 m raster maps by ESA WorldCover 2020, based on Sentinel-1 and Sentinel-2 data (Zanaga et al. 2021). We reclassified the land uses as (1) forest, (2) non-forested natural areas, (3) pastures and grassland, (4) croplands, (5) urban areas, (6) water bodies and wetlands, and calculated their cover. Additionally, we calculated the minimum distance from the colony to water bodies (6) and urban areas (5). As linear water bodies that could be used as water sources, like rivers and small streams, are underestimated in raster maps, we corrected the value of minimum distance to land class water bodies and wetlands (6) using the EU-Hydro layers

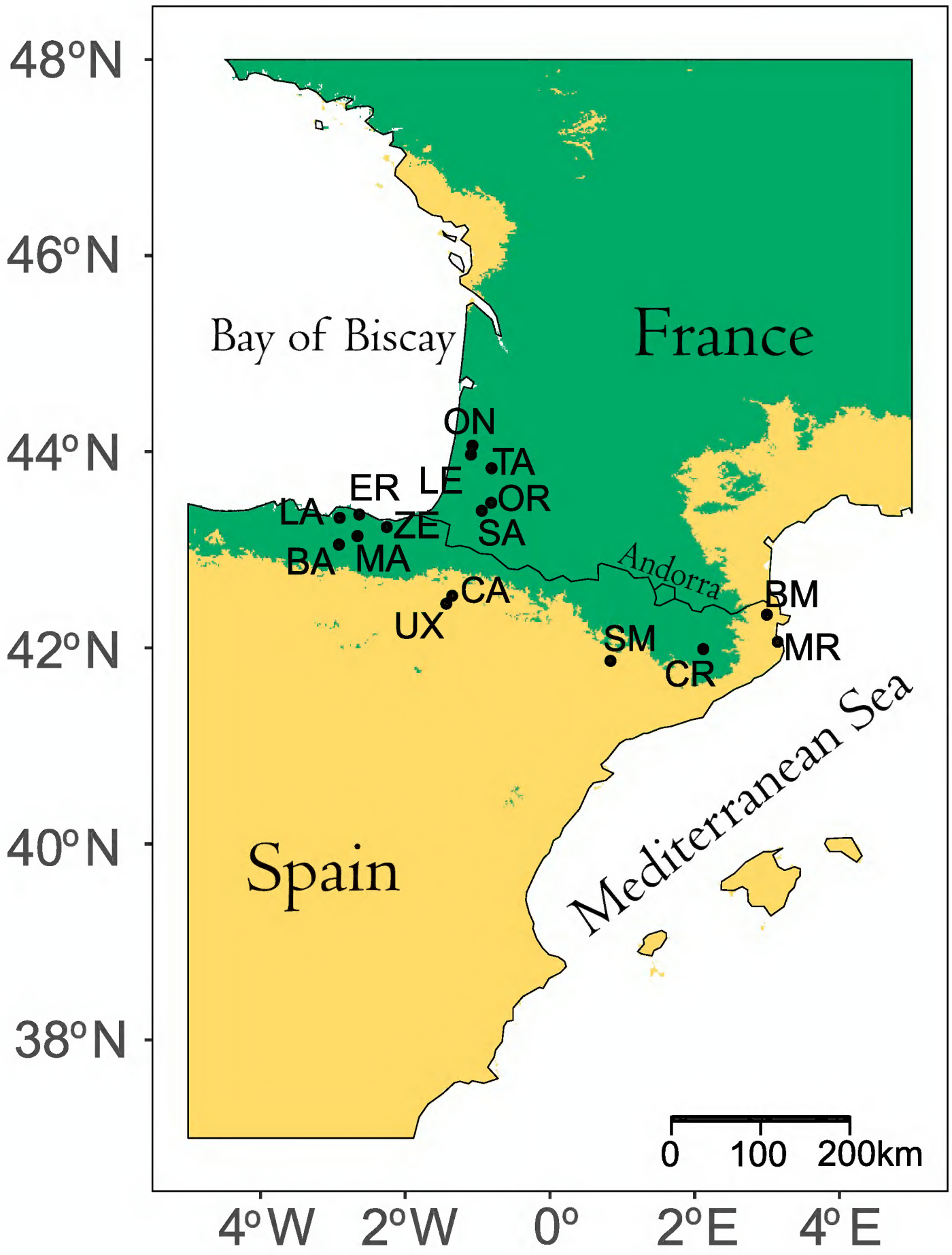

Figure 1. Locations of the maternity colonies of *M. emarginatus*. Yellow: dry summer climate, i.e., driest summer month has less than 40 mm of rain. Green: wet summer climate, i.e., driest month of the summer has over 40 mm of rain.

(European Environment Agency 2019). Finally, we used official cattle census data from Spain and France (INE 2020; DRAAF Nouvelle Aquitaine 2020; DRAAF Occitaine 2020) to estimate the density of total cows within each 10 km radius around roosts, as well as the mean number of cows per livestock farm.

Sample collection, laboratory processing and bioinformatic process

Faecal samples were collected on two different days from each colony in 2020; one at the end of May (spring) and another at the beginning of July (summer). The samples were processed with those presented in Vallejo et al. (2023), following identical methodologies and criteria. For each day and colony sampled, hereon sampling event, we passively collected up to 40 samples each consisting of 2–6 pellets (approximate weight 30–60 mg). DNA from each sample was

extracted using DNeasy PowerSoil Kit and DNeasy PowerSoil Pro Kit (Qiagen) following manufacturer's protocol with some modifications (see supplementary material 1 from Vallejo et al. 2023) Each extraction round included 23 samples and one negative extraction control.

Following DNA extraction, all samples and extraction blanks were amplified using primer set FWH1, as described by Vamos et al. (2017), to amplify DNA from both potential prey items and bats. For the amplification process we used 10 µl of Qiagen multiplex PCR kit, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 7 µl H2O and 1 µl of DNA. Cycling conditions started with 15 min at 95 °C for polymerase activation, followed by 40 cycles of 94 °C (30 s) - 45 °C (45 s) - 72 °C (2 m), and finished with 10 minutes at 72 °C. Amplification products were then purified using magnetic beads.

Four individual metagenomic libraries were built following official Illumina protocols (Illumina 2013), and one control was added per library at this stage. In the first library, two controls were added. During this process, index sequences and Illumina adapters were attached using Nextera XT v2. A different combination of forward and reverse markers was used in each sample to allow their differentiation. Amplification success was checked by migrating the product in an agarose gel before purifying again and pooling all the samples at equal molarities for sequencing. Sequencing was performed in Illumina MiSeq (500 cycle v2 kit), in four separate runs. For this study, we used 505 samples, which were processed together with more *M. emarginatus* samples included in Vallejo et al. (2023), and one sample belonging to *Miniopterus schreibersii*, which did not belong to any study. In total, we sequenced 940 biological samples, 39 extraction blanks and five library blanks with no DNA template.

Bioinformatic analyses were done with VSEARCH (Rognes et al. 2016) and Cutadapt (Martin 2011) to process raw sequences, merge paired-end reads, trim the primer sequences and cluster them into operational taxonomic units (OTUs) at the 97% similarity threshold. These were compared to online databases using the *blastn* function in BLAST+ (Camacho et al. 2009) to access the GenBank dataset, and Boldigger-cline (Buchner and Leese 2020) to access the BOLD database. We accepted all matches above a 98% identity and an e-value lower than $1e^{-20}$, when provided. The results were curated using a custom script in R version 4.4.0 (R Core Team 2024), followed by a manual curation to ensure a single taxon matched per OTU. We classified all identifications as belonging to the predator, environmental contamination, potential prey species absent from the study area, or potential prey species present in the study area. Bioinformatic procedures, and the parameters used in the process are detailed in Vallejo et al. (2023, appendix 1).

All subsequent analyses were performed in R version 4.4.0 (R Core Team 2024), and all visualizations were done using *ggplot2* (Wickham 2016). In order to ensure that all samples used in the analysis belonged to the target species, samples were discarded if more than 10% of all the reads identified as a bat belonged to species other than *M. emarginatus*. On the other hand, extraction and library blanks were examined to account for potential pervasive contamination events regarding prey items but no taxa were removed from the analysis at this point. However, under the presumption that OTUs with low abundances are likely sources of mistakes and contamination events, OTUs with less than 0.5% reads were removed in each sample (Drake et al. 2021).

Diet description

Only those OTUs classified as potential prey items found in the study area were used for the diet analysis. We converted all relative read abundance values to binary data, and built diet tables at the species, family, and order levels. To make a general description of the diet, we calculated the frequency of occurrence (FOO) and weighted percentage of occurrence (wPOO) values of all dietary items at the species, family and order level as described by Deagle et al. (2019). Additionally, we used six dietary variables to explore their relationship with landscape variables: first order Hill numbers at the species, family and order levels, and spider species richness per sampling event, which were calculated using R package *hilldiv* (Alberdi 2019); and wPOO values of diurnal flies (as family Muscidae) and spiders (as order Araneae) per sampling event.

Statistical analyses

We first tested the normality of all our dietary variables with the Shapiro-Wilk test. We applied the arcsine square root transformation to dietary proportion variables to improve their fit (Gotelli and Ellison 2004). We explored differences in the dietary metrics between the two sampling seasons (spring and summer) using paired t-tests. Then, we explored relationships between the dietary metrics and land cover variables, per sampling season.

Before the analysis, we used the square root arcsine transformation on all land cover variables, the log transformation on the number of cattle heads, and the mean number of cattle heads per operation. To explore the ordination of the bat colony association with land cover we performed a PCA. All landscape variables were standardized prior to data analysis to ensure that the scales were comparable. Then, we extracted the first three principal components, which were then related to dietary variables in a multiple regression using function *glm* of package *stats* in R (R Core Team 2024).

Finally, we modelled diurnal fly consumption using a two-part or hurdle model, which are commonly employed when the response variable is zero-inflated. This approach assumes that two different biological processes are at play; in our case, one would cause the absence of flies in the diet, and the other would influence their frequency of occurrence (Zuur et al. 2009). The choice of this model was guided by the *a priori* hypothesis that the presence of cow sheds and cattle could be a prerequisite for the availability and therefore the consumption of flies by *M. emarginatus*. Accordingly, we implemented a hurdle model using function *hurdle* in package *pscl* (Zeileis et al. 2008), specifying (i) the probability of flies being consumed by the colony, modelled as a binary response to the landscape variables, and (ii) the relative importance of flies in the diet, modelled as the wPOO values ranging from 1 to100, and rounded to the nearest integer. To account for over-dispersion, we selected a zero truncated negative binomial distribution. As explanatory variables, we included the raw number of cattle surrounding the colony and transformed and standardized values for the proportion of forested and urban areas. We also included collection season as a factor. We built all possible combinations of models and chose the best one based on AICc values using the *dredge* function from package *MuMIn* (Bartoń 2023).

Results

Landscape variability in the study area

The selected colonies were predominantly surrounded by forests, both native and plantations, as well as grasslands and croplands at varying proportions. In some Mediterranean colonies other types of natural areas, mainly shrublands, were also noticeably present (Fig. 2A). The number of cows at a 10 km radius was also highly variable between the selected colonies, and so was the mean number of cows per farm, reflecting the variability in livestock management between colonies (Fig. 2A).

The first three axes of the PCA accounted for 80% of the total variation in the data (Fig. 2B). The first axis explained 51% of the total variability and separated the western and northern colonies, characterized by higher proportions of tree cover and grassland, from those colonies with greater cropland cover, larger areas of other natural habitats and higher numbers of cows per farm, mainly located in the south-eastern part of the study area. This axis also correlated with the natural oceanic-Mediterranean climatic gradient present in the study area. The second axis explained 19% of the variation and was correlated with higher levels of urbanization and greater numbers of cows in a 10 km radius. The third axis, which was not portrayed in the PCA plot, explained 9% of the variation and separated colonies according to their proximity to urban and coastal areas.

Selection samples and OTUs

The four MiSeq runs generated over 44 million paired-end reads, and a mean of 44876 reads per sample. In colony CR an unexpectedly high number of *Miniopterus schreibersii* individuals were found in the roost that year, so collection

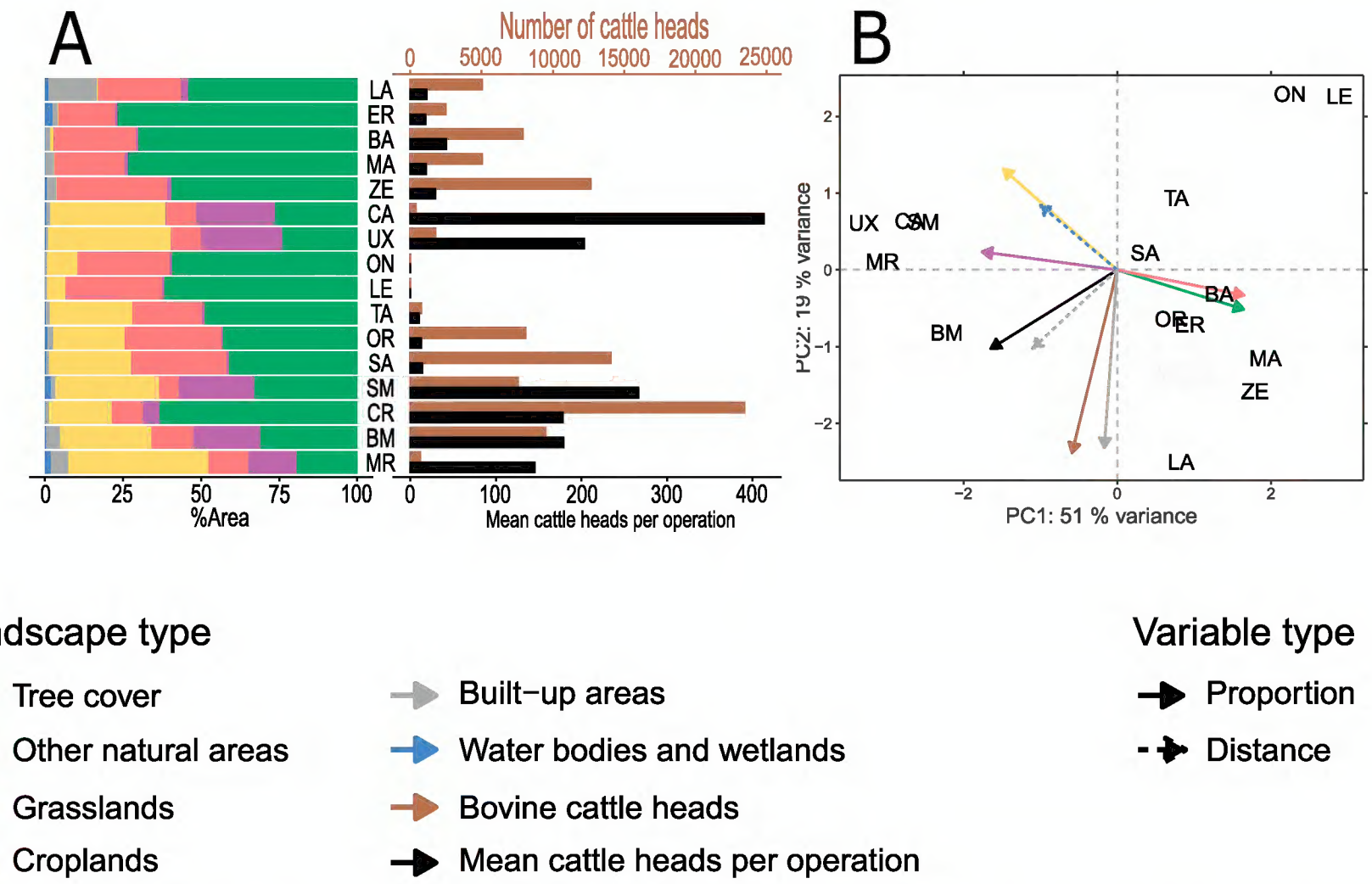

Figure 2. A bar chart depicting the proportion of land cover types in a 10 km radius around the colony, and an estimation of the number of bovine cattle heads and mean number of cattle heads per operation in the same area **B** PCA of the landscape variables calculated for all the maternity colonies.

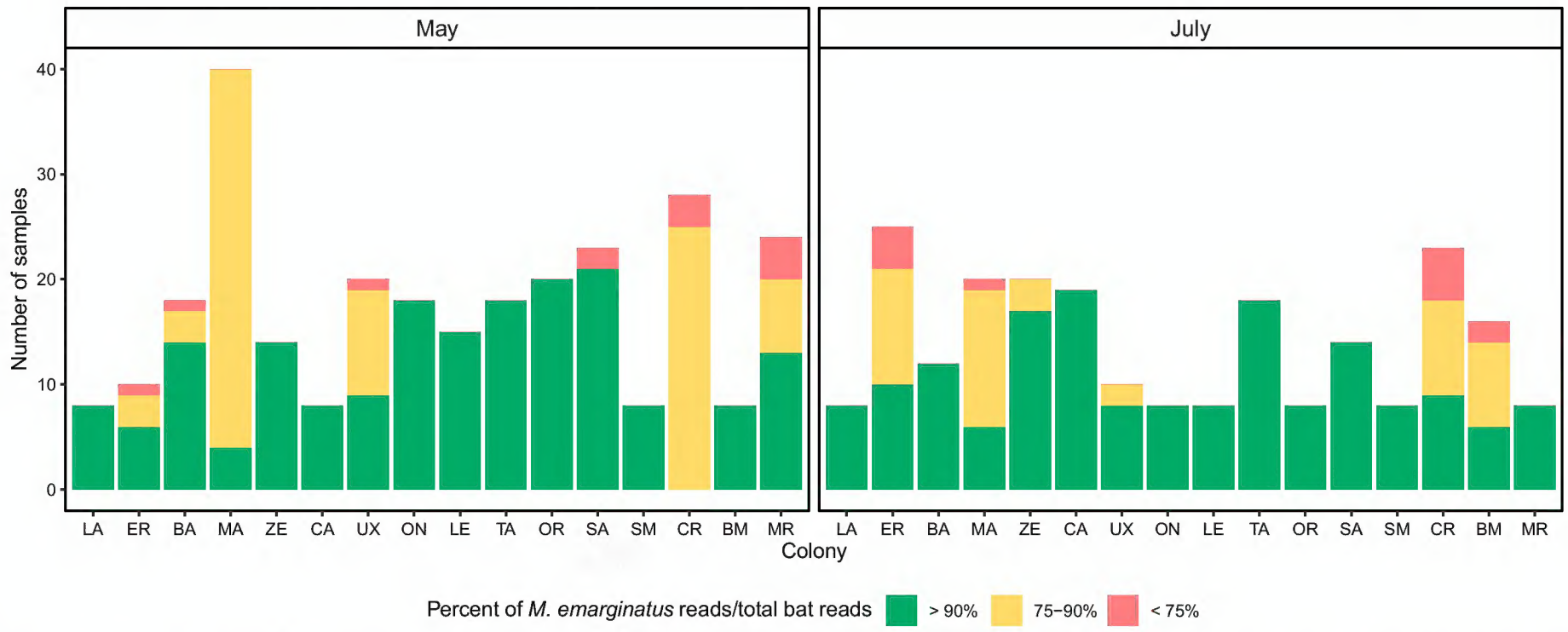

Figure 3. Frequency of samples processes in each colony and date. Colour refers to sample quality regarding amount of DNA corresponding to *Myotis emarginatus*: green > 90%; yellow 75-90%, red < 75%. Only Green samples were chosen for the diet analysis.

of samples was impossible in spring, and very challenging in summer. As a result, this colony was removed from further analysis. In colony MA bats moved within the roost during the night, resulting in very few samples belonging to *M. emarginatus* being collected. The roost in LA was empty in the first week of July (summer), so we used samples from mid-July instead. In total, 8941 OTUs from 505 samples were compared against GenBank and BOLD Systems databases, which comprise the full dataset (Suppl. material 2). As expected, some samples from mixed colonies contained DNA of co-occurring bat species, and as a result, we removed 154 samples from further analysis as per the criteria explained in the previous section (Fig. 3). After excluding non-eligible samples due to external contamination, improper amplification, or sequencing error related to prey items, 320 samples were left for the dietary analysis (Suppl. material 3).

The selected samples contained a total of 15.3 million raw reads, and a mean of 43729 reads per sample. After processing, 14 million reads were assigned to 7366 OTUs, of which only 825 had a frequency of occurrence exceeding 0.5% in any sample. Ten of them (55% of the total sequence reads) belonged to the predator, *M. emarginatus*, 103 (5% of reads) were classified as environmental contamination, 318 (24% of reads) were identified as potential prey items, and eight belonged to potential prey species that are not found in the study area (0.4% of reads). The remaining 386 OTUs (15.6% of reads) did not match with any sequence in the reference databases at the 98% identity threshold.

Diet description

A total of 253 prey species belonging to 97 families and 16 orders were identified. Most prey species were only identified in a single sample (147 prey species) or in two samples (40 prey species). Spiders (Araneae) were the most consumed prey order (wPOO: 52%, FOO: 83%). Moreover, spiders exhibited the highest wPOO values in 22 sampling events out of 30 (Fig. 4) and were consumed more in July (Fig. 5). The second most consumed prey order was Diptera (wPOO: 29%, FOO: 62%). The diet was completed by Coleoptera, Lepidoptera, Hemiptera, Ephemeroptera and Odonata, in decreasing order of wPOO.

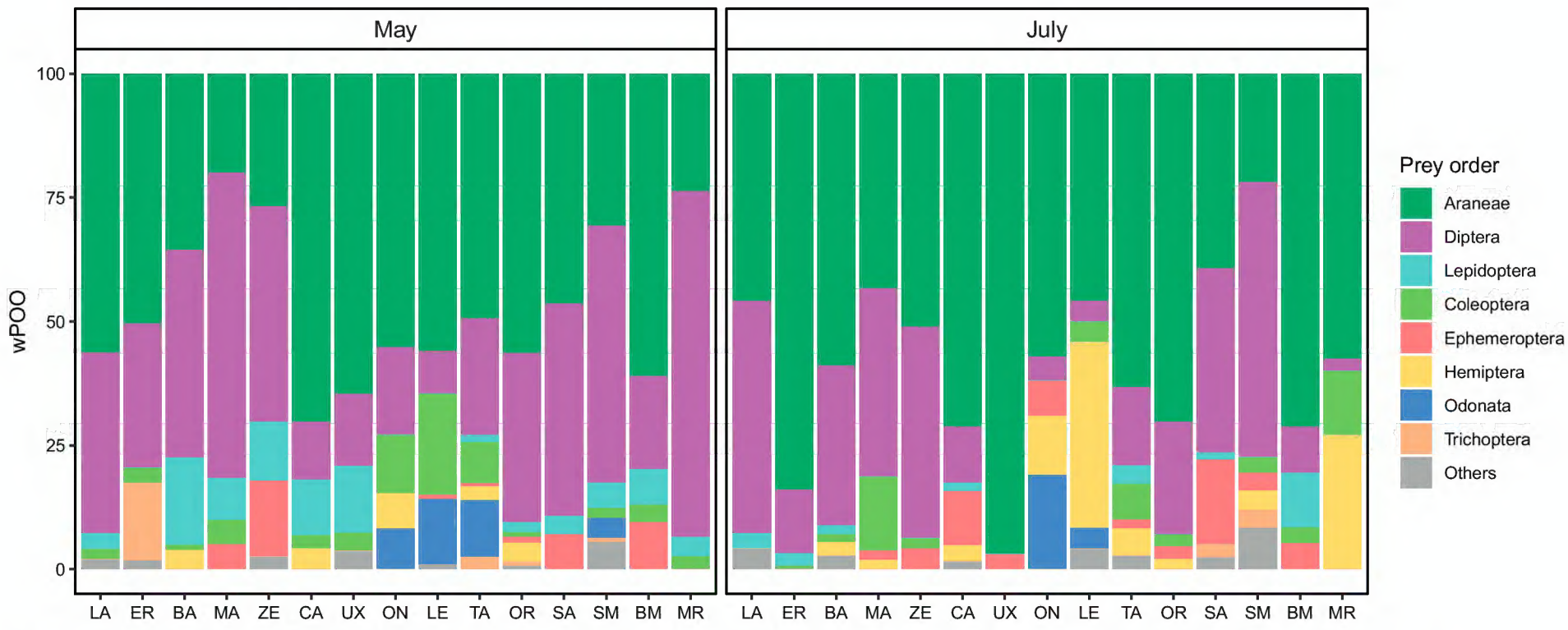

Figure 4. Weighted percentage of occurrence (wPOO) values of each of the sampling events at the order level.

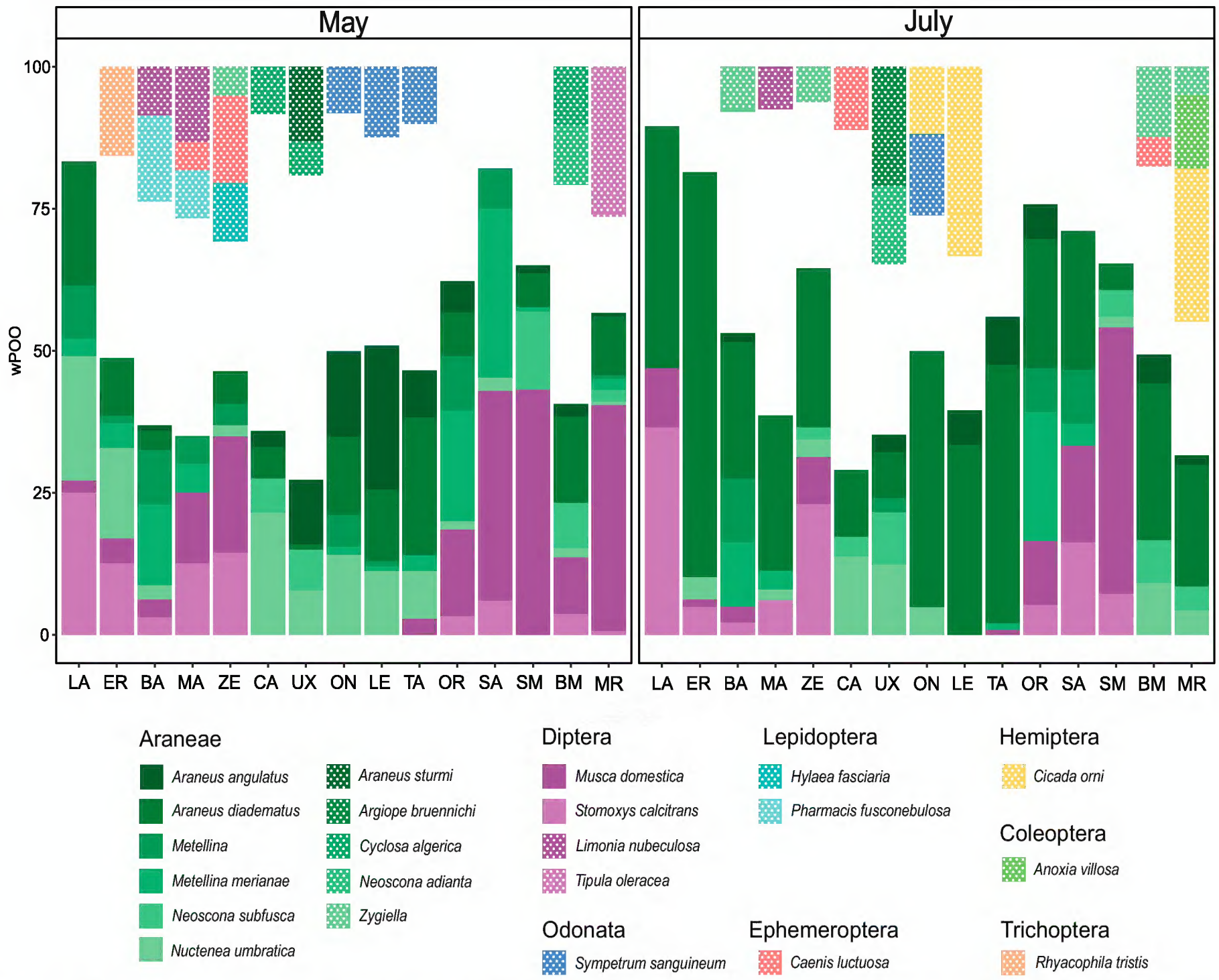

Figure 5. Weighted percentage of occurrence (wPOO) of prey. The eight species with more than 10% frequency of occurrence overall are displayed in the bottom of the graph and with solid colours. Additionally, those species with wPOO higher than 10% at each sampling event are displayed in the top of the graph with dotted colours.

Eight prey species had a FOO value over 10% (n > 32) (Table 1). These include five species and one genus of orb-web building spiders and two diurnal pest flies associated with livestock farming. Collectively, these eight species

Table 1. Prey species that were identified in more than 32 samples (FOO %10), and their global frequency of occurrence (FOO) and weighted percentage of occurrence (wPOO).

ORDER	FAMILY	SPECIES	FOO	wPOO
Araneae	Araneidae	*Araneus diadematus*	179	20.15
Diptera	Muscidae	*Musca domestica*	95	11.23
Araneae	Araneidae	*Nuctenea umbratica*	66	4.97
Araneae	Tetragnathidae	*Metellina merianae*	54	4.22
Araneae	Araneidae	*Araneus angulatus*	50	4.16
Diptera	Muscidae	*Stomoxys calcitrans*	48	5.03
Araneae	Araneidae	*Metellina* sp.	42	2.82
Araneae	Araneidae	*Neoscona subfusca*	33	2.31

comprised 27% to 75% of the diet in any sampling event, as measured by wPOO. Among the spiders, *Araneus diadematus* was the most consumed (wPOO: 20%, FOO: 56%), particularly in July. House flies (*Musca domestica*) were the most consumed diurnal fly (wPOO: 11%, FOO: 30%), especially by bats from the Mediterranean colonies. Whereas consumption of stable flies (*Stomoxys calcitrans*) was greater in more oceanic colonies. Beyond these eight taxa, 14 additional ones were locally noteworthy, contributing to more than 10% wPOO in at least one sampling event (Fig. 5). This category includes several large species like, *Argiope bruennichi* (Araneae), *Sympetrum sanguineum* (Odonata), *Anoxia villosa* (Coleoptera) or *Cicada orni* (Hemiptera).

Seasonal variation

Dietary diversity was higher in May at all taxonomical levels (Fig. 6), albeit at the order level the difference was only marginally significant (Species: t = 3.073, df = 14, p-value = 0.0083; Family: t = 2.854, df = 14, p-value = 0.0127; Order: t = 2.113, df = 14, p-value = 0.0529). Spider species richness (t = 2.353, df = 14, p-value = 0.0338) was also significantly higher in May. However, the proportion of spiders in the diet increased significantly from May to July (t = -2.745, df = 14, p-value = 0.0158). The wPOO of diurnal flies, counted as species belonging to

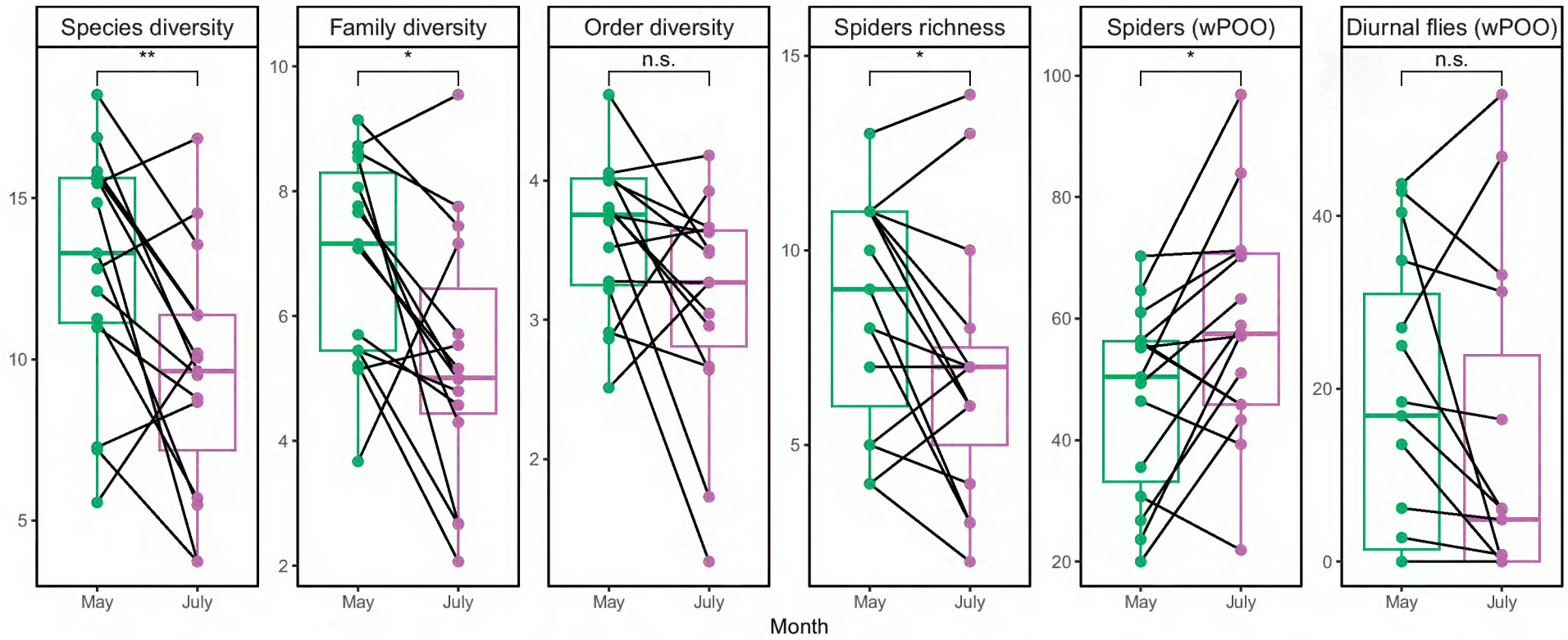

Figure 6. Seasonal differences between dietary metrics. Lines link observations from the same colony. Significance values of the paired t-test are represented (n.s. = non-significant, * = 0.01 < p-value < 0.05, ** = p-value < 0.05).

the family Muscidae, did not change significantly between sampling seasons (t = 1.379, df = 14, p-value = 0.1895) and the exclusion of the colonies where no diurnal fly consumption was detected did not alter the results (t = 1.3959, df = 10, p-value = 0.193).

Relationship with landscape

Spider richness in both seasons was negatively associated with the first axis of the landscape PCA (May: PC1 = -0.8992, t = -3.091, df = 14, p-value = 0.0103; July: PC1 = -1.2570, t = -4.422, df = 14, p-value = 0.0010). In May, the wPOO of diurnal flies was significantly related to the second PC (PC2 = -0.12767, t = -2.551, df = 14, p-value = 0.0269), whereas in July, the relationship was only marginally significant (PC2 = -0.11894, t = -2.060, df = 14, p-value = 0.0639). No other dietary metric showed significant associations with the PCA axes.

The best hurdle model included the raw number of cows, the percentage of forested area and the percentage of urban area in the negative binomial component, while only the raw number of cows was selected in the binomial section. The probability of consuming diurnal flies increased significantly with more cattle heads near the colony (est = 0.0004, z = 3.198, p-value = 0.0014). However, once fly consumption occurred, the wPOO value was not related to the number of cattle around the colony (est = $8.647 \cdot 10^{-5}$, z = 1.338, p-value = 0.1808). Instead, the importance of diurnal flies in the diet was significantly shaped by the percentage of forested areas (est = -0.3779, z = -2.536, p-value = 0.0112) and the percentage of urban areas (est = 0.2717, z = 2.136, p-value = 0.0327) (Fig. 7).

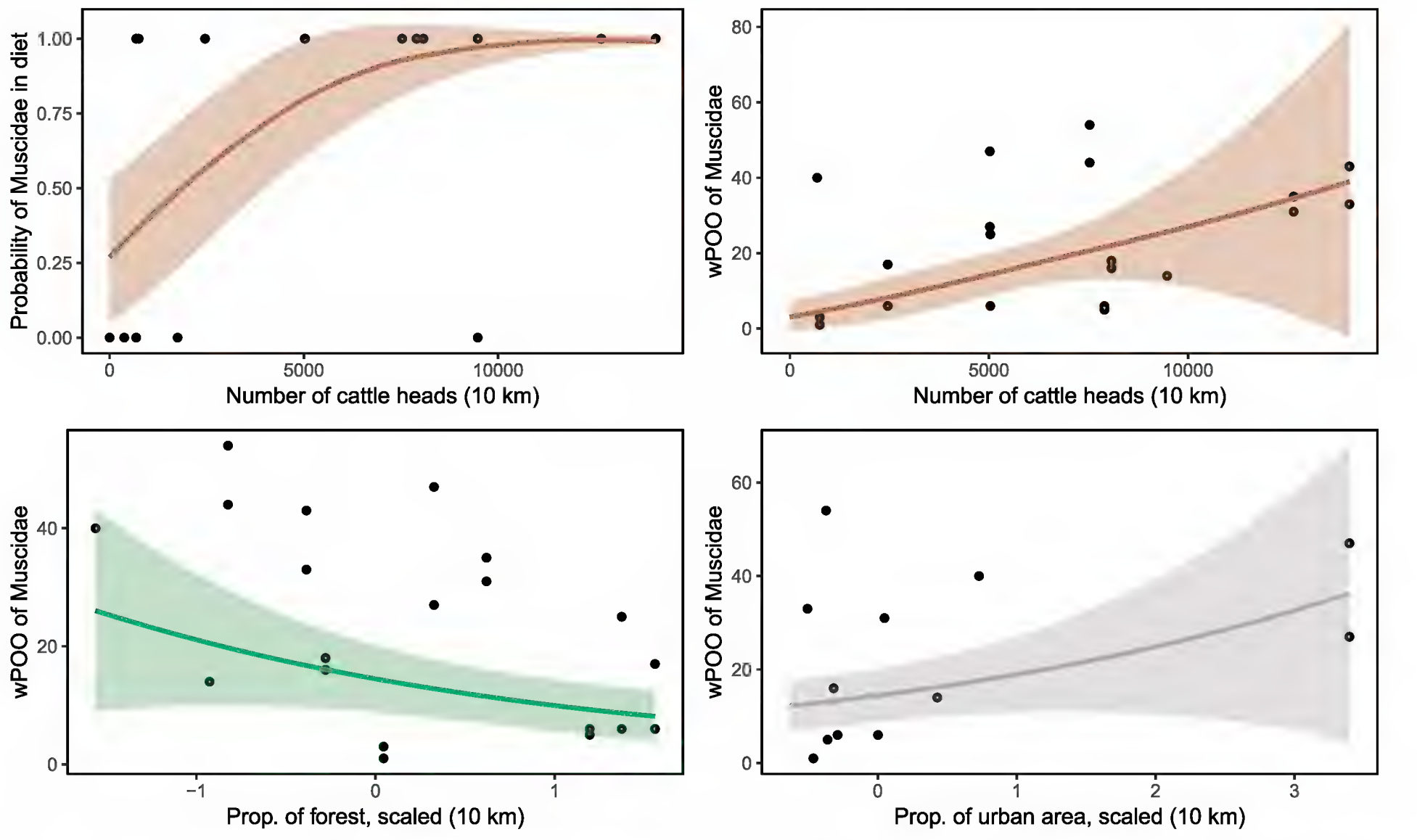

Figure 7. Marginal prediction lines of the parameters of the hurdle model.

Discussion

General description of the diet: globally and locally important prey items

Our findings underscore the significant role of both orb-web building spiders (e.g. Araneidae, Tetragnathidae) and diurnal flies (Muscidae) as the primary prey type of the notch-eared bat in southwestern Europe. These taxa collectively accounted for 68% of the total wPOO, and between 42% and 97% of the wPOO per sampling event, which reinforces *M. emarginatus'* reliance on gleaning for foraging, as both are likely hunted while resting. Non-volant and/or diurnal prey are commonly found in the diets of other gleaning bat species which, thanks to their slow and manoeuvrable flight style, can catch prey from a variety of substrates while they are immobile. Adopting this foraging style allows bats to forage successfully even when availability of flying insects drops, such as during periods of adverse weather (Burles et al. 2009) or winter (Hope et al. 2014).

In our study area, the recently described species *Myotis crypticus* and *Myotis escalerai* seem to be the closest to *M. emarginatus* in terms of trophic niche, as they are all medium-sized bats that forage in forests, specialize in gleaning, and consume non-volant and diurnal prey, including numerous orb-web building spider species (Novella-Fernandez et al. 2020; Razgour et al. 2021). In northern Europe *Myotis nattereri* has shown similar foraging behaviour (Andreas et al. 2012; Hope et al. 2014), and, in addition, has also been observed consuming diurnal pest flies inside of cattle sheds (Siemers et al. 2012). In addition to gleaning for prey in natural areas, foraging inside of buildings further ensures prey availability for bats during adverse weather conditions and periods of low flying insect availability (Pir and Dietz 2018).

However, *M. emarginatus* is the only species that seems to prey preferentially on orb-web building spiders across a wide geographical area. Our results on the dietary composition of the notch-eared bat are consistent with previous studies (Krull et al. 1991; Benda et al. 2010; Steck and Brinkmann 2006; Goiti et al. 2011; Kervyn et al. 2012; Vallejo et al. 2019, 2023; Vescera et al. 2024), and highlight the robustness of the notch-eared bat's dietary preferences across a wide geographical range, and reinforces its reliance on gleaning for foraging.

Dietary diversity at all taxonomic levels, as well as spider richness, was lower in July than in May, in accordance with previous studies (Vallejo et al. 2023; Vescera et al. 2024). In temperate areas, both flying insects and spiders show a peak of abundance in late summer (Hails 1982; Hsieh and Linsenmair 2012), a pattern also observed for diurnal pest flies in the western part of our study area (Valbuena-Lacarra and Saloña-Bordas 2010). Thus, the decline in dietary diversity may be a response to overall higher prey availability, allowing bats to be more selective and specialize in the most profitable prey items (Emlen 1966).

The consistent dietary preference for orb-web building spiders across different regions suggests they are profitable prey for *M. emarginatus*. In fact, the spider species identified in this study are active throughout the *M. emarginatus´* breeding season (Hsieh and Linsenmair 2012), they are often abundant (Nyffeler and Bonte 2020) and reach substantial size (Roberts 1996). On top of that, specializing in orb-web building spiders can help *M. emarginatus* avoid competition from other non-gleaning bat species. Similarly, the overabundance of flies such as *S. calcitrans* and *M. domestica* in cattle sheds is presumed to be the reason why bats visit them to forage (Dekker et al. 2013; Vescera et al. 2024). These species are inactive at

night, so they are only accessible to gleaning bats (Siemers et al. 2012). Both prey items are highly profitable for *M. emarginatus*; especially for females who need a constant and predictable food supply to rapidly rear their pups during their short stay at the nursery colonies (Spitzenberger and Weiss 2021; McGuire and Boyles 2024).

M. emarginatus exploits other types of resources as well. We found occasional but abundant genetic remains of large arthropods like *Cicada orni* (Hemiptera), *Sympetrum sanguineum* (Odonata) or *Anoxia villosa* (Coleoptera). Big arthropods like these are staple prey of larger bat species in Europe, such as *Eptesicus serotinus* (Tiede et al. 2020), *Rhinolophus ferrumequinum* (Tournayre et al. 2020), *Nyctalus noctula* (Lindecke et al. 2021) or *Myotis* (Zahn et al. 2021), but our results show that smaller bats can also consume them. Whereas hunting and handling bigger insects is costlier for bats, a single individual can be highly profitable, making them attractive to predators (Fenton 1990). Given their size and behaviour, it is likely that *M. emarginatus* gleans these prey items while they are stationary on vegetation (Hykel et al. 2017; Sanborn et al. 2011). These species or similar ones have been recorded in small quantities in the diet of other European gleaners using similar molecular methods (e.g., Andriollo et al. 2021; Novella-Fernandez et al. 2021). In the case of the notch-eared bat, our results suggest that their consumption is most likely tied to temporary population peaks. Nonetheless, the importance of big prey items should be considered with caution, as their size could enhance their detectability on DNA metabarcoding studies compared to smaller taxa (Elbrecht et al. 2017).

Forested areas, Mediterranean ecosystems and their relationship to spider consumption

Our study identified several common forest spiders, such as *Araneus diadematus*, *Araneus angulatus*, *Nuctenea umbratica* or *Neoscona subfusca*, as some of the most common prey. These and other orb-web building spiders thrive in forested habitats and areas with complex vegetation, where they have ample structures to anchor their webs (Dennis et al. 2015). This aligns with the well-documented spatial behaviour of the foraging notch-eared bat, often described as a forest gleaner that primarily selects native deciduous woodlands for hunting (Dietz and Pir 2021). These orb-web building spiders, and specifically *Araneus diadematus*, are suffering a decline in their population in central Europe as a response to the widespread loss of flying insects (Nyffeler and Bonte 2020), which could have cascading effects on the diet and foraging success of *M. emarginatus*.

Our results showed that the wPOO of spiders in the diet was not significantly related to any landscape variable, suggesting that bats eat spiders similarly across the wide geographical area under study, irrespective of the proportion of forest available around the roost. Nonetheless, spider richness in the diet was significantly higher in Mediterranean colonies. This could be attributed to a richer entomofaunal community associated with Mediterranean landscapes. However, it could also reflect a broader range of foraging habitats used by *M. emarginatus* in these colonies, thus encountering more species. Mediterranean type colonies had a lower proportion of forested areas but, in turn, had higher proportions of scrublands and other natural semi open areas, which Mediterranean *M. emarginatus* are known to hunt in (Goiti et al. 2011). In fact, orb-web building spiders commonly found on dryer and more open areas, such as *Cyclosa algerica* or

Neoscona adianta, and lower vegetation and grasses, such as *Argiope bruennichi*, were important prey in the surveyed Mediterranean colonies. A similar dietary pattern can be expected in other areas of the Mediterranean.

Consumption of flies is dependent on livestock farming, but not exclusively

Our analysis showed that the probability of consumption of diurnal flies such as the house fly (*Musca domestica*) and the stable fly (*Stomoxys calcitrans*) by *M. emarginatus* colonies changes with the presence of cows around roosts. The consumption of diurnal flies was none or negligible in areas where cattle farming was unimportant, thus, the alleged dependence of northern breeding *M. emarginatus* on livestock farming (Steck and Brinkmann 2006; Kervyn et al. 2012; Dietz et al. 2013, Pir and Dietz 2018) did not apply to our study area. Furthermore, our hurdle model estimates that bats are likely to consume diurnal flies when the number of cattle heads exceeds 2000. However, once the consumption of diurnal flies is established, their relative importance in the diet as measured by wPOO is not related to cattle numbers, aligning with the results reported by Vescera et al. (2024).

The absence of a linear fly-cattle relationship could be explained by several factors. First, *M. emarginatus* likely searches actively for places with high fly concentrations like cattle sheds (Vescera et al. 2024), which may result in a high local consumption of flies even in places with relatively low livestock densities. Second, while the number of cattle heads around the colony can be a good proxy for the abundance of pest flies, it does not necessarily reflect their availability for bats. For example, dairy cows are usually housed in sheds during the night, supporting larger densities of flies in the sheds and encouraging foraging by *M. emarginatus* (Dekker et al. 2013). Extensive meat livestock farms, on the other hand, may be less attractive to *M. emarginatus*, potentially explaining the lower fly consumption observed in the westernmost colonies of our study.

Furthermore, our results suggest that the wPOO of diurnal flies is not solely dependent on the livestock farming activity around the colony. The percentage of forested area, for instance, was negatively related to the wPOO of flies, likely because they are a decent source of other prey such as spiders, moths, or beetles, increasing the dietary diversity and hampering the dominance of pest flies. In contrast, the percentage of urban areas showed the opposite effect, a decrease of prey in wild areas due to increased urbanization (Raupp et al. 2010) could be forcing bats to forage on cattle sheds. This highlights the behavioural plasticity of *M. emarginatus* and its ability to rely on a new type of resource (inactive flies inside buildings) by exploiting its original foraging behaviour (gleaning for non-flying prey in cluttered environments).

The predator-prey interaction between *M. emarginatus* and diurnal pest flies is also noteworthy because both stable flies and house flies are epiparasitic, and may significantly harm cattle, transmit viral and bacterial diseases, and reduce milk production (González et al. 2022). The management of these pests is complex, especially in modern operations (Gerry and Murillo 2019). The notch-eared bat positively responds to their availability at least to some extent, and thus presents an interesting opportunity for the adoption of integrated pest management strategies. However, the complex relationship between fly consumption, geography, climate and landscape characteristics pose a challenge to the quantification and extrapolation of the predation pressure that a single *M. emarginatus* colony can exert.

Strengths and limitations of DNA metabarcoding

As high-throughput sequencing has become more affordable, its popularity has surged. Advanced technologies now provide sequencing depths that allow for the processing of hundreds of samples without compromising species coverage, facilitating extensive sampling designs like ours. DNA metabarcoding, in some contexts, allows for the identification of prey items to the species level (Alberdi et al. 2017). This, in the present study, has allowed us to confirm that *M. emarginatus* relies on gleaning for most of its foraging, and also that spiders common in semi-open areas are important prey in the Mediterranean.

Additionally, non-invasive sampling allows for the collection of more samples with little impact on the animals compared to capturing individual bats, but it is also prone to sample degradation and contamination (Naef et al. 2023). The sensitivity of DNA metabarcoding can amplify these effects, but careful sample handling in the field and in the laboratory, and rigorous data curation mitigate such risks (Ficetola et al. 2016). Additionally, dietary data from DNA metabarcoding may include false positives, especially for predators like *M. emarginatus* at high trophic levels (Tercel et al. 2021). In our study, distinguishing secondary predation (DNA of the spider's prey) from true prey was challenging, prompting the use of strict bioinformatic filters and careful dataset interpretation.

A significant limitation of DNA metabarcoding, particularly amplicon metabarcoding, is its lack of reliable quantitative accuracy, even when employing generalist primers (Krehenwinkel et al. 2017). As a result, many authors advise against using retrieved sequence count information, or relative read abundances, for the description of diet composition, in favour of presence/absence data (Elbrecht and Leese 2015). Metrics derived from occurrence data, such as wPOO, can provide a good summary of dietary composition, but it is unlikely that they would represent the ingested biomass accurately (Deagle et al. 2019). This hampers efforts to assess the predation pressure bats like *M. emarginatus* exert on specific pest species. More specific methods, such as those described by Baroja et al. (2021), are better suited for this purpose, though none have yet been applied to study predation by *M. emarginatus* on diurnal pest flies.

Conclusions

Advancements in High Throughput Sequencing and a non-invasive sampling methodology allowed us to perform one of the most extensive dietary studies on a European bat species on an intermediate geographical scale.

The main prey items of the notch-eared bat remain consistent over a large geographically extensive and climatically diverse range. Orb-web building spiders are the preferred prey type in the southern half of the western distribution (Goiti et al. 2011; Vallejo et al. 2019, 2023). Yet, their consumption does not depend on landscape features such as the presence of forested areas. Furthermore, the known habitats of some spider species suggest that natural habitats other than deciduous forests are a good source of prey for this bat in the Mediterranean, so conservation efforts should go beyond the conservation of forested areas. Instead, management for the conservation of *M. emarginatus* should be focused on broad scale preservation of a variety of native habitat types; resulting in the conservation of their associated arthropod communities.

This would ensure availability of orb-web building spiders, as well as other insects that might be hunted opportunistically (Frick et al. 2024).

The importance of pest flies for the survival of *M. emarginatus* should not be underestimated either, especially in northern colonies (Pir and Dietz 2018; Vescera et al. 2024). Our results show that, when available, bats will take advantage of pest flies, therefore restricting the access of bats to cowsheds will negatively impact pest fly profitability (Dietz and Pir 2021). If suitable sources of pest flies disappear, our results suggest that *M. emarginatus* colonies will be most vulnerable if the surrounding natural habitats are also degraded.

Despite the rising number of studies on the dietary preferences of the notch-eared bat, it is seldom noted as a predator of livestock pest flies beyond the restricted realm of bat studies. In-depth interdisciplinary studies on the efficacy of bats to control pest fly populations along human-induced geographical and environmental gradients are required. This way, integrated pest management strategies in Europe could improve the conservation status of wild bat populations and the welfare of farm animals alike. This bat species is a good research subject candidate to address such questions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: IG, NV. Data curation: NV. Formal analysis: NV. Funding acquisition: IG. Investigation: NV, LOH. Methodology: MA, NV. Software: NV. Supervision: IG. Visualization: NV. Writing - original draft: NV. Writing - review and editing: MA, JA, IG, LOH.

Author ORCIDs

Nerea Vallejo https://orcid.org/0000-0002-1478-1536

Miren Aldasoro https://orcid.org/0000-0002-7393-3878
Lander Olasagasti https://orcid.org/0000-0002-1408-0991
Joxerra Aihartza https://orcid.org/0000-0003-0882-8964
Inazio Garin https://orcid.org/0000-0001-7085-5352

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information. The raw sequences belonging to this study have been published in the ENA repository, under study accession number PRJEB90539 / ERP173542.

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Supplementary material 1

Description of the colonies

Authors: Nerea Vallejo, Miren Aldasoro, Lander Olasagasti, Joxerra Aihartza, Inazio Garin

Data type: csv

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Link: https://doi.org/10.3897/mbmg.9.144371.suppl1

Supplementary material 2

List of all OTUs found in the samples studied. It includes sequence, taxonomic assignation and number of reads in each sample

Authors: Nerea Vallejo, Miren Aldasoro, Lander Olasagasti, Joxerra Aihartza, Inazio Garin

Data type: csv

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Supplementary material 3

Samples included in the analysis and information regarding colony and date of collection, and quality of the sample regarding contamination from co-occurring bat species

Authors: Nerea Vallejo, Miren Aldasoro, Lander Olasagasti, Joxerra Aihartza, Inazio Garin

Data type: csv

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